

Rapid agonist mediated phosphorylation of the metabotropic glutamate receptor 1 α by protein kinase C in permanently transfected BHK cells

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Abstract Clonal BHK cells permanently transfected with the metabotropic glutamate receptor 1 α (mGluR1 α), which is coupled to phospholipase C, were used to study the phosphorylation state of the receptor. Cells were labelled with $^{32}\text{PO}_4^{3-}$, lysed, the receptor immunoprecipitated with specific anti-peptide antibodies and the immunoprecipitates analysed by SDS-PAGE followed by autoradiography. A significant basal level of receptor phosphorylation was observed which was rapidly and transiently increased in response to agonist activation of the receptor. This agonist effect was found to be dose dependent with a rapid time course and could be abolished by the specific PKC inhibitor Ro318220, suggesting that PKC was responsible for the agonist mediated phosphorylation of the receptor.

Key words: Metabotropic glutamate receptor; Phosphorylation; Protein kinase C; Desensitization; Inositol trisphosphate; Diacylglycerol

1. Introduction

The metabotropic glutamate receptors (mGluRs) constitute a distinct family of G-protein linked receptors with seven putative membrane spanning domains. They are unusual in that they are much larger than, and appear to have little sequence homology with any of the other G-protein linked receptors [1]. To date seven subtypes of this receptor family have been cloned (mGluR1–7), with differing pharmacological properties and tissue distributions. These seven receptor subtypes may be further categorized into three subgroups on the basis of sequence homology, agonist selectivity and signal transduction pathway. One subgroup consists of mGluR1 and mGluR5. The former exhibits three splice variants termed mGluR1 α , mGluR1 β and mGluR1 γ and the latter two splice variants namely mGluR5 α and mGluR5 β [2–7]. This group of receptors is coupled to phospholipase C (PLC) which catalyzes inositol phospholipid (IP) hydrolysis, resulting in the production of both diacylglycerol (DAG) which stimulates protein kinase C (PKC), and inositol trisphosphate (IP₃) which induces release of calcium from intracellular stores [2–6]. Another subgroup consists of

mGluR2 and mGluR3, both of which inhibit forskolin stimulated cAMP formation in transfected chinese hamster ovary (CHO) cells [1,8]. The third subgroup is made up of mGluR4, mGluR6 and mGluR7, all of which are sensitive to L-AP4 and are also coupled to the inhibition of forskolin stimulated cAMP formation in transfected CHO cells [8–11]. Recent evidence has suggested the existence of a further subtype mGluR8, which is highly homologous with mGluR7 [12].

Studies carried out in cerebellar granule cells [13] and in rat hippocampal slices [14], have demonstrated that mGluR mediated IP hydrolysis may be inhibited by direct stimulation of PKC using phorbol esters. Co-incubation of phorbol esters with PKC inhibitors not only reverses this inhibition but also causes a potentiation of the response [14]. Similar results have been obtained with the metabotropic glutamate receptor mGluR1 α expressed in permanently transfected baby hamster kidney (BHK) cells [15], and this has led to the suggestion that agonist induced activation of PKC may result in rapid receptor desensitization by a negative feedback mechanism involving a PKC mediated phosphorylation event. Further evidence for this comes from studies of the mGluR mediated DAG response in cerebrocortical nerve terminals [16]. This response was found to undergo rapid and reversible desensitization and was strongly inhibited by direct stimulation of PKC with phorbol ester. Similarly the recovery of the DAG response could be prevented by addition of the phosphatase inhibitor okadaic acid.

Studies into the mechanisms of rapid desensitization in G-protein coupled receptors have mainly concentrated on the β_2 -adrenergic receptor. This has been shown to undergo rapid desensitization following agonist stimulation due to phosphorylation of the receptor by the combined actions of protein kinase A (PKA) and the β -adrenergic receptor kinase [17–19]. Phosphorylation is believed to result in functional uncoupling of the receptor from G α_s , causing a decrease in the sensitivity of the agonist stimulated cAMP response. Little however is known about the mechanisms underlying the rapid desensitization of PLC linked receptors such as mGluR1 and mGluR5. Agonist mediated phosphorylation of both the α_{1b} -adrenergic receptor and the m3-muscarinic receptor has been demonstrated, and there is evidence to suggest that this may be involved in their desensitization, although no direct link between these two processes has yet been established [20–23]. Furthermore, the kinase or kinases responsible for this phosphorylation have not been positively identified, although PKC appears to have been ruled out following investigations with the specific PKC inhibitor Ro318220, in which application of maximal doses of this compound failed to inhibit the agonist induced phosphorylation of either of these receptors [21,23].

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Abbreviations: mGluR, metabotropic glutamate receptor; BHK, baby hamster kidney; PLC, phospholipase C; IP, inositol phospholipid; DAG, diacylglycerol; IP₃, inositol trisphosphate; PKC, protein kinase C; PKA, protein kinase A; β -ARK, β -adrenergic receptor kinase; CHO, chinese hamster ovary; DCI, dichloroisocoumarin; PdBu, phorbol-12,13-dibutyrate; DMEM, Dulbecco's Modified Eagle Medium; FCS, foetal calf serum; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

The metabotropic glutamate receptors possess a number of C-terminal serine and threonine residues which may provide sites for potential regulatory phosphorylation [1]. In view of the established importance of PKC in regulating the responses of some of these receptors, there appears to be a genuine prospect that agonist mediated phosphorylation of PLC linked mGluRs could provide a mechanism for rapid, reversible receptor desensitization. In the following study we demonstrate for the first time that a PLC-coupled receptor mGluR1 α expressed in permanently transfected BHK cells is rapidly and transiently phosphorylated by PKC in response to agonist, and that the time course of phosphorylation is sufficiently rapid for this to represent a plausible mechanism for rapid desensitization of the receptor.

2. Materials and methods

2.1. Materials

Baby hamster kidney (BHK) cells permanently transfected with the metabotropic glutamate receptor mGluR1 α , anti-peptide anti-serum A4 to mGluR1 α together with the corresponding A4 peptide were all kindly provided by Dr. E. Mulvihill. This cell line has been extensively characterized in the literature with respect to expression of the receptor, its agonist selectivity and signal transduction pathway [15,24,25]. The antibody A4, raised against an intracellular sequence (EFVYER-EGNTEDEL) from mGluR1 α has also been extensively characterised [26–28]. Sodium dihydrogen [32 P]orthophosphate (1–150 mCi/mg) was obtained from Amersham. Methatretaxate, *S. Aureus* insoluble Protein A suspension, O-phenanthroline, L-glutamic acid and phorbol 12,13-dibutyrate were obtained from Sigma. L-Quisqualic acid and (RS)-4-carboxy-3-hydroxyphenylglycine were obtained from Tocris Cookson. E-64 and dichloroisocoumarin (DCI) were obtained from Calbiochem. Geneticin G-418 sulphate was obtained from Gibco. The specific PKC inhibitor Ro318220 [29] was kindly donated by Dr. T.J. Hallam, Roche Products Ltd.

2.2. Tissue culture

BHK cells stably expressing the T45A clone of mGluR1 α were grown in 25 cm² flasks containing 5 ml Dulbecco's Modified Eagle Medium (DMEM), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5% foetal calf serum (FCS), 1 μ M methatretaxate and 0.5 mg/ml G-418 sulphate at 37°C and in an atmosphere of 5% CO₂.

2.3. Affinity purification of A4 anti-serum

Crude A4 anti-serum was affinity purified against the A4 peptide using the ProtOn Kit (Multiple Peptide Systems, San Diego, CA, USA) and using the manufacturers recommended protocol.

2.4. [32 P]Phosphate labelling of cells, immunoprecipitation and SDS-PAGE

Cell cultures (2 ml) were grown to pre-confluence (70–90%) in 6-well plates (Falcon). The cells were washed gently with DMEM (2 ml) containing no phosphate, no FCS and no L-glutamine, and then incubated with 100 μ Ci/ml [32 P] PO_4^{3-} for 2 h in 1 ml of the same medium at 37°C and 5% CO₂. Agonists and other compounds were added to the medium as indicated. The labelling medium was then rapidly removed and the cells lysed for 1 min on ice with 1 ml RIPA detergent buffer containing 0.5% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EGTA, 1 mM MgCl₂, 150 mM NaCl, 1 mM Na₃VO₄, 0.05% (w/v) NaN₃, 50 mM Tris-HCl (pH 7.4). The lysing medium also contained the protease inhibitors o-phenanthroline (20 μ g/ml), DCI (22 μ g/ml) and E-64 (18 μ g/ml). Cell lysates were centrifuged at 14,000 rpm at 4°C for 10–15 min in an Eppendorf microfuge and the supernatant was immunoprecipitated with 1 μ g/ml affinity purified A4 antibody for 1 h at 4°C. A 50 μ l volume of a 1:1 suspension of *S. aureus* insoluble Protein A made up in RIPA buffer was then added to the supernatant and samples were rotated at 30 rpm for a further 1 h at 4°C. The Protein A-IgG-receptor immunocomplex was pelleted at 14,000 rpm then resuspended and washed 3 times in 1 ml RIPA buffer and once in 1 ml of 50 mM Tris-HCl, pH 7.4. The pellet was then resuspended in 30 μ l

of sample buffer containing 20 mM DTT, 4% SDS, 20% glycerol and 100 mM Tris-HCl (pH 6.8), and the immunoprecipitated protein eluted by incubation at 70°C for 2 min. Previous work carried out in this laboratory has demonstrated that Tran 35 S-labelled mGluR1 α is quantitatively and specifically immunoprecipitated by the antibody A4 as a 150 kDa protein [28]. Eluted proteins were resolved by discontinuous SDS polyacrylamide gel electrophoresis using 3% polyacrylamide stacking gels and 7.5% polyacrylamide running gels. The gels were dried and then exposed to Kodak X-Omat film at –70°C for 15–20 h. Where indicated, band intensities were quantified by gray-scale densitometry using the software package Optilab (Graftek).

3. Results

BHK cells permanently transfected with mGluR1 α were labelled with [32 P] PO_4^{3-} , the cells lysed and the receptor immunoprecipitated with A4 antibody as described in section 2. Analysis of the immunoprecipitate by SDS-PAGE followed by autoradiography revealed a significant basal level of receptor phosphorylation (Fig. 1a), which was substantially increased in response to a 2 min stimulation with either 100 μ M L-quisqualate or 1 mM L-glutamate (Fig. 1b). Pre-incubation for 10 min with the specific PKC inhibitor Ro318220 (10 μ M) reduced receptor phosphorylation below basal levels (Fig. 1c), and also abolished the agonist induced increase in phosphorylation (Fig. 1d). Taken together these data suggest that mGluR1 α is rapidly phosphorylated by PKC in response to agonist stimulation, and that there is a significant basal level of phosphorylation, probably resulting from a constitutive activation of PKC in these cells. A 10 min incubation with a supra-maximal dose of the PKC activator phorbol 12,13-dibutyrate (PdBu) (1 μ M) caused a large increase in receptor phosphorylation, confirming that mGluR1 α is indeed a substrate for PKC (Fig. 1e). In a separate experiment this phorbol ester induced phosphorylation was shown to be completely ablated when 1 μ M PdBu was co-incubated with 10 μ M Ro318220 (Fig. 1f–h). The large quantity

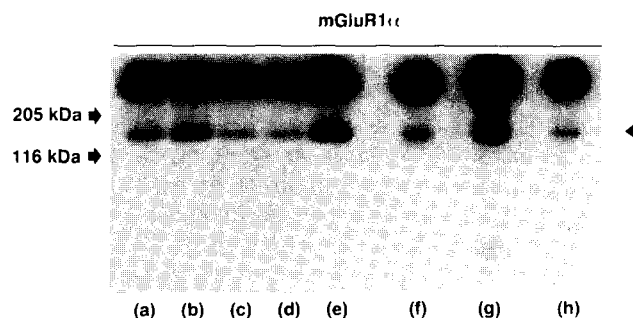


Fig. 1. Agonist mediated phosphorylation of mGluR1 α expressed in permanently transfected BHK cells. Cells were grown in identical 2 ml cultures in a 6-well plate and isotopically labelled with [32 P] PO_4^{3-} for 2 h as described in section 2. The cells in individual wells were then: (a) left untreated as a control; (b) treated with 100 μ M L-quisqualate for 2 min; (c) treated with 10 μ M Ro318220 for 10 min; (d) treated with 10 μ M Ro318220 for 10 min followed by 100 μ M L-quisqualate for a further 2 min (still in the presence of Ro318220); and (e) treated with 1 μ M PdBu for 10 min. In a separate experiment cells in individual wells were (f) left untreated as a control, (g) treated with 1 μ M PdBu for 10 min and (h) co-incubated with 1 μ M PdBu and 10 μ M Ro318220 for 10 min. The extracellular medium was then rapidly removed, the cells lysed and the receptor immunoprecipitated and analysed by SDS-PAGE followed by autoradiography as described in section 2. The figure shows data from two separate, representative experiments both of which were carried out 3 times. The same results were observed in parallel experiments in which cells were stimulated with 1 mM L-glutamate.

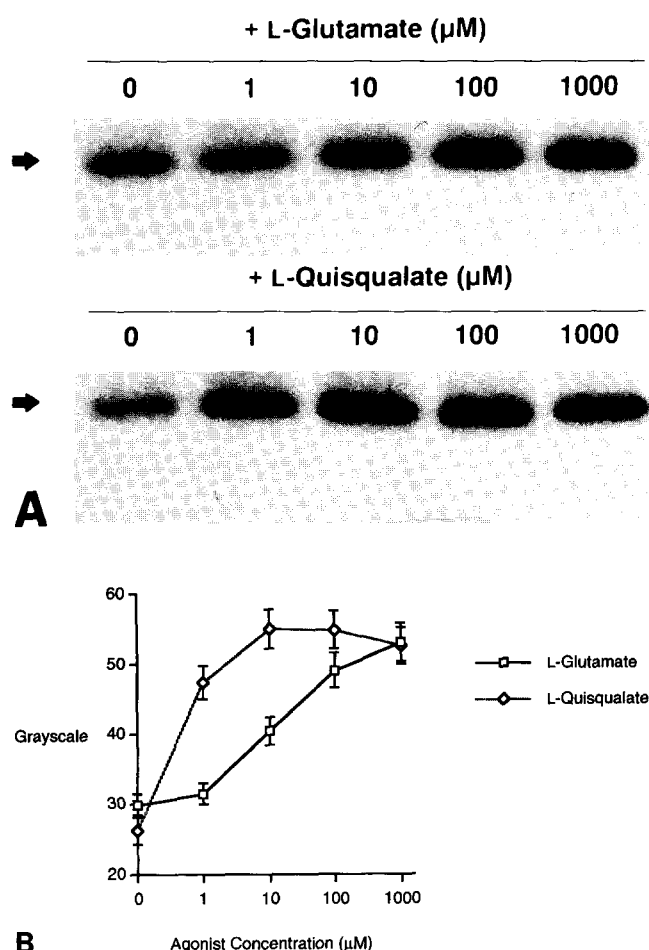


Fig. 2. Dose-response curves for agonist mediated phosphorylation of mGluR1 α expressed in permanently transfected BHK cells. (A) Cells were grown in identical 2 ml cultures in two 6-well plates and isotopically labelled with [32 P] PO_4^{3-} for 2 h as described in section 2. The cells in individual wells were then either left untreated as a control or treated with doses of L-glutamate or L-quisqualate in the range 1, 10, 100 and 1000 μ M for a period of 2 min. The extracellular medium was then rapidly removed, the cells lysed and the receptor immunoprecipitated and analysed by SDS-PAGE followed by autoradiography as described in section 2. (B) The variation in intensity of the 150 kDa band arising from application of different doses of agonist was quantified by grayscale densitometry using arbitrary units. The figure shows data from a single representative experiment that was carried out 3 times.

of material at the top of the gel has also been observed in immunoprecipitates from Tran 35 S-labelled cell lysates and represents aggregated receptor [28]. Although the level of phosphorylation of this material corresponds with that of the 150 kDa receptor 'monomer', work carried out previously in this laboratory has shown that this aggregate cannot be resolved fully from underlying non-specifically immunoprecipitated material [28]. Consequently our studies have concentrated on the 150 kDa form of the receptor which is clearly resolved on SDS gels.

In order to discount the possibility of non-specific effects resulting from the use of such high concentrations of agonist, the cells were pre-labelled with [32 P] PO_4^{3-} and then stimulated for 2 min with concentrations of 1, 10, 100 and 1000 μ M of both L-glutamate and L-quisqualate. The receptor was analysed by SDS-PAGE followed by autoradiography and the variation in

intensity of the 150 kDa band was quantified by gray-scale densitometry. These studies demonstrated that the degree of agonist mediated phosphorylation is not only dependent on the agonist concentration (Fig. 2A) but also generated dose response curves for L-glutamate and L-quisqualate that are characteristic for these agonists acting at mGluR1 α and which show the expected greater efficacy of L-quisqualate as an agonist [15] (Fig. 2B). The increase in mGluR1 α phosphorylation associated with 100 μ M L-glutamate and 5 μ M L-quisqualate could be reduced but not completely blocked by the selective mGluR1 α antagonist (*RS*)-4-carboxy-3-hydroxyphenylglycine (data not shown). The failure to block completely these responses probably reflects the high concentrations of agonist used and the relatively low affinity of this competitive antagonist [30]. Taken together these results show that the agonist mediated increase in mGluR1 α phosphorylation is due to their effects on the receptor and not a consequence of non-specific interactions arising from treating the cells with high doses of L-glutamate and L-quisqualate.

The time course of the agonist mediated phosphorylation of the receptor was also studied. Cells pre-labelled with [32 P] PO_4^{3-} were stimulated with 100 μ M L-quisqualate or 1 mM L-glutamate for periods of 1, 2, 5, 15 and 30 min. The immunoprecipitates were analysed by SDS-PAGE followed by autoradiography, and the variation in intensity of the 150 kDa band was quantified by grayscale densitometry (Fig. 3). It was found that the level of phosphorylation rose rapidly within the first minute following addition of the agonist, peaked at about 2 min and then returned back to basal levels approximately 15–30 min later. These results demonstrate that the receptor is rapidly and transiently phosphorylated by PKC in response to agonist activation, and that this transient phosphorylation displays a relatively short time course.

4. Discussion

The inhibitory effect of PKC activation on the responses of certain PLC linked metabotropic glutamate receptors has been studied by a number of groups [13–16]. The study by Thomsen et al. [15] was carried out using the same BHK cells transfected with mGluR1 α that we have used in this investigation. In similar experiments we have confirmed both the agonist mediated activation of mGluR1 α expressed in these cells and inhibition of the receptor mediated responses resulting from pre-activation of PKC that Thomsen et al. reported ([15]; Alaluf et al., unpublished data). Together these data suggest that PKC may be involved in regulating mGluR1 α desensitization in these cells, and the finding in this study that mGluR1 α is rapidly and transiently phosphorylated by PKC in response to agonist is consistent with this, and represents a potential mechanism by which such desensitization may occur.

The rapid agonist induced increase in receptor phosphorylation is consistent with the rapid translocation of PKC to the plasma membrane observed in striatal neurons following activation of mGluRs [31]. Similarly, the time course of mGluR1 α phosphorylation observed in this study is remarkably consistent with the time course of recovery of the desensitized mGluR DAG response measured in cerebrocortical nerve terminals by Herrero et al. [16]. In that study, no second response to agonist was observed until 10 min after the initial response, by which time we have shown that the level of receptor phosphorylation

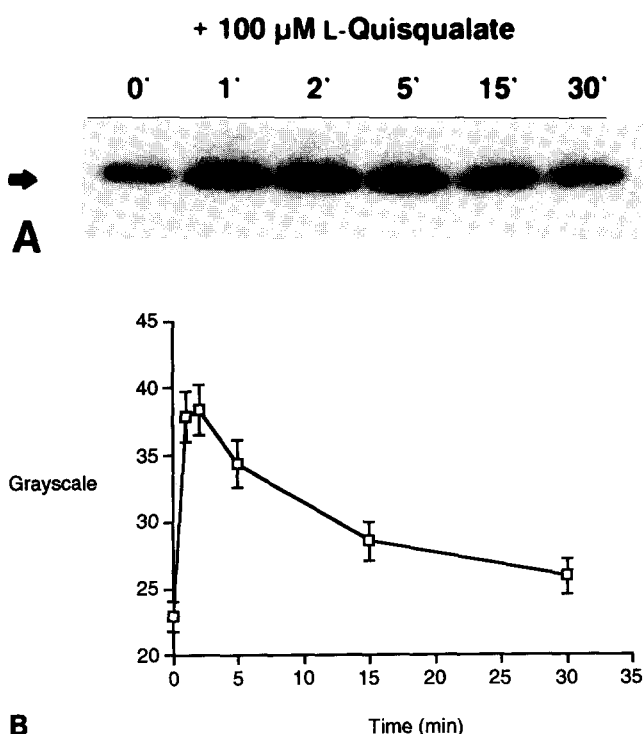


Fig. 3. Time course of agonist mediated phosphorylation of mGluR1 α expressed in permanently transfected BHK cells. (A) Cells were grown in identical 2 ml cultures in a 6-well plate and isotopically labelled with [32 P] PO_4^{3-} for 2 h as described in section 2. The cells in individual wells were then either left untreated as a control or treated with 100 μ M L-quisqualate for periods of 1, 2, 5, 15 and 30 min as indicated. The extracellular medium was then rapidly removed, the cells lysed and the receptor immunoprecipitated and analysed by SDS-PAGE followed by autoradiography as described in section 2. The figure shows data from a single representative experiment that was carried out 3 times. (B) The variation in intensity of the 150 kDa band during the time course was quantified by gray-scale densitometry using arbitrary units. A similar time course was observed in parallel experiments in which cells were stimulated with 1 mM L-glutamate.

has peaked and is falling. This second response gradually returned to normal 20 min following the first addition of agonist, by which time receptor phosphorylation has almost returned to basal levels. It appears therefore that agonist mediated phosphorylation of mGluR1 α does constitute a credible mechanism by which the receptor stimulated DAG response may be rapidly desensitized.

The presence of a significant basal level of phosphorylation was at first thought to be due to the presence of trace quantities of L-glutamate in the [32 P] PO_4^{3-} labelling medium. Although L-glutamate is rigorously excluded during the [32 P] PO_4^{3-} labelling period, it is present in the normal growth medium due to the presence of 5% foetal calf serum (FCS). However, we have found that pre-incubating the cells with the selective mGluR1 α antagonist (*RS*)-4-carboxy-3-hydroxyphenylglycine (1 mM) in FCS-free medium for 24 h before labelling with [32 P] PO_4^{3-} has no effect on the basal level of receptor phosphorylation (unpublished data). This, together with the fact that the PKC inhibitor Ro318220 was able to reduce receptor phosphorylation below basal levels suggests that there is a constitutive activation of PKC in these cells. This is not surprising when one considers that PKC may be stimulated by many different path-

ways, not least by the various mitogens and growth factors which are always present under normal culture conditions [32].

In conclusion, we have observed a rapid, transient phosphorylation of mGluR1 α by PKC in response to agonist activation. The degree of phosphorylation is dependent on the agonist concentration and we suggest that it may represent a plausible mechanism for rapid receptor desensitization. Current investigations are being directed towards identifying the site or sites of phosphorylation in order to determine more clearly if phosphorylation is indeed responsible for the regulation of receptor responses.

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